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11/22/00 JC946 U.S. PTO

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 1.53(b))

Attorney Docket No.	11520.0222
First Inventor	Suga, et al
Title	Catalytic RNAs With .....
Express Mail Label No.	EK837074856US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

1. ■ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ■ Applicant claims small entity status. See CFR 1.27.
3. ■ Specification [*Total Pages* / 25 /]
(preferred arrangement set forth below)
- Descriptive title of the invention
- Cross Reference to Related Applications
- Statement Regarding Fed sponsored R&D
- Reference to sequence listing, a table, or a computer program listing appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

4. ■ Drawing(s) *(35 USC 113)* [*Total Sheets* / 5 /]
5. ■ Oath or Declaration [*Total Pages* / 4 /]
a. ■ Unexecuted Copy
b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☐ Application Data Sheet. See 37 CFR 1.76
7. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program *(Appendix)*
8. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
a. ■ Computer Readable Form (CRF)
b. ☐ Specification Sequence Listing on:
i. ☐ CD-ROM or CD-R (2 copies); or
ii. ■ paper
c. ■ Statements verifying identity of above copies

jc714 U.S. PTO 09/721414 11/22/00

ACCOMPANYING APPLICATION PARTS

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
11. ☐ English Translation Document *(if applicable)*
12. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
13. ☐ Preliminary Amendment
14. ■ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ■ Other: Check in the amount of $555

17. **If a CONTINUING APPLICATION**, *check appropriate box and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:*
☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of the prior application No: __/_____

Prior application information: Examiner:_____ Group/Art Unit: _____

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

18. CORRESPONDENCE ADDRESS

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"Express Mail" Mailing Label Number EK837074856US Date of Deposit November 22, 2000

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Ranjana Kadle
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Ranjana Kadle
Name
Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suga, et al
Serial No.: Not yet assigned
Filed: Herewith
For: Catalytic RNAs With Aminoacylation Activity

LETTER

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants declare that the Paper Copy of the Sequence Listing and the Computer Readable Copy filed with the above-referenced patent application are the same.

Respectfully Submitted,

Ranjana Kadle
Reg. No. 40,041

Hodgson, Russ, Andrews, Woods & Goodyear, LLP
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November 22, 2000

BFLODOCS:468979_1 (@1V701)

PTO/SB/17 (08/00) Approved for use through 10/31/2002, OMB 0651-0032

FEE TRANSMITTAL for FY 2000

Patent Fees are subject to annual revision.

Application Number	
Filing Date	November 22, 2000
First Named Inventor	Suga, et al
Examiner Name	
Group/Art Unit	
Attorney Docket Number	11520.0222

TOTAL AMOUNT OF PAYMENT	($) 555.00

1. ■ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number: 08-2442
Deposit Account Name: Hodgson, Russ, Andrews, Woods & Goodyear, LLP

■ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

■ Applicant claims small entity status. See 37 CFR 1.27.

2. ■ **Payment Enclosed:**
■ Check ☐ Credit Card ☐ Money Order ☐ Other

FEE CALCULATION

1. FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	$ 355
106	320	206	160	Design filing fee	$
107	490	207	245	Plant filing fee	$
108	710	208	355	Reissue filing fee	$
114	150	214	75	Provisional filing fee	$
				SUBTOTAL (1)	$ 355

2. EXTRA CLAIM FEES

	Extra Claims	Fee from below	Fee Paid
Total Claims	/11/ -20** =	/0/ x	/0/ = $ 0
Independent Claims	/8/ - 3** =	/5/ x	/40/ = $ 200
Multiple dependent	/ /	x / /	= $

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim if not paid
109	80	209	40	**Reissue independent claims over original patent
110	18	210	9	**Reissue claims in excess of 20 and over original patent
				SUBTOTAL (2) $ 200

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	$
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	$
139	130	139	130	Non-English specification	$
147	2,520	147	2,520	For filing a request for reexamination	$
112	920*	112	920*	Requesting Publication of SIR prior to Examiner Action	$
113	1,840*	113	1,840*	Requesting Publication of SIR after Examiner Action	$
115	110	215	55	Extension for reply within first month	$
116	390	216	195	Extension for reply within second month	$
117	890	217	445	Extension for reply within third month	$
118	1,390	218	695	Extension for reply within fourth month	$
128	1,890	228	945	Extension for reply within fifth month	$
119	310	219	155	Notice of Appeal	$
120	310	220	155	Filing a brief in support of an appeal	$
121	270	221	135	Request for oral hearing	$
138	1,510	138	1,510	Petition to institute a public use proceeding	$
140	110	240	55	Petition to revive - unavoidable	$
141	1,240	241	620	Petition to revive - unintentional	$
142	1,240	242	620	10 advance copies Utility issue fee (or reissue)	$ $
143	440	243	220	Design issue fee	$
144	600	244	300	Plant issue fee	$
122	130	122	130	Petitions to the Commissioner	$
123	50	123	50	Petitions related to provisional applications	$
126	240	126	240	Submission of Information Disclosure Statement	$
581	40	581	40	Recording each patent assignment per property (times number of properties)	$
146	710	246	355	Filing a submission after final rejection(37 CFR 1.129(a))	$
149	710	249	355	For each additional invention to be examined (37 CFR 1.129(b))	$

*Reduced by basic filing fee paid **SUBTOTAL (3)** $ 0

SIGNATURE: Ranjana Kadle

Ranjana Kadle Reg. No. 40,041

DATE: November 22, 2000 **Telephone:** (716) 848-1628

Express Mail" Mailing Label Number EK837074856US Date of Deposit November 22, 2000

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Ranjana Kadle
Name

Ranjana Kadle
Signature

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CATALYTIC RNAs WITH AMINOACYLATION ACTIVITY

This application claims the priority of US provisional application number 60/167,331 filed on November 24, 1999 and US provisional application number 60/214,382 filed on June 28, 2000, the disclosure of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to the field of catalytic RNA molecules, and more particularly to catalytic RNA molecules having the ability to aminoacylate tRNA-like molecules in *cis* or in *trans*.

BACKGROUND OF THE INVENTION

Proteins containing non-natural amino acids hold great promise for biomedical and therapeutic purposes. Such amino acids may be particularly useful in the structural and functional probing of proteins, construction of peptide libraries for combinatorial chemistry, and in proteomics. However, the synthesis of such proteins has not heretofore been easy. In the translation system that is known to occur currently in nature, genetic coding is carried out by aminoacyltRNA synthetases (ARSs). They exist in 20 different forms, each of which specifically catalyzes the esterification of a single amino acid to its cognate tRNA isoacceptor, thereby directly connecting the amino acid with its corresponding anticodon triplet. Because misacylation of noncognate amino acids to tRNAs causes misincorporation of amino acids into cellular proteins which can be fatal to their intracellular activity, the fidelity of the aminoacylation reactions by the ARSs must be extremely high. To achieve this important task, the ARSs use very sophisticated mechanisms to selectively recognize the cognate amino acids and tRNAs. The recognition determinants of tRNAs are diverse ranging from the anticodon loop to the acceptor-Ti~C stem and the

phosphate-ribose backbone. Because of these complexities, engineering of ARSs with desired specificities toward nonnatural tRNAs and amino acids has not been achieved. As a result attention has turned to the nucleic acids.

For many years, nucleic acids were considered to be only informational molecules. However, the pioneering work of Cech and coworkers (Cech, 1987, Science, 236:1532-1539; McCorkle et al., 1987, Concepts Biochem. 64:221-226) demonstrated the presence of naturally occurring RNAs that can act as catalysts (ribozymes). However, although these natural RNA catalysts have only been shown to act on ribonucleic acid substrates for cleavage and splicing, recent development of artificial evolution of ribozymes has expanded the repertoire of catalysis to various chemical reactions. For example, RNAs have been reported to catalyze phosphodiester cleavage on DNA (Beaudry et al., 1992, Science, 257:635), hydrolysis of aminoacyl esters (Piccirilli et al., 1992, Science, 256:1420-1424), self-cleavage (Pan et al., 1992, Biochemistry, 31:3887), ligation of an oligonucleotide with a 3'0H to the 5'triphosphate end of the catalyst (Bartel et al., 1993, Science, 261:1411-1418), biphenyl isomerase activity (Schultz et al., 1994, Science, 264:1924-1927), and polynucleotide kinase activity (Lorsch et al., 1994, Nature, 371:31-36).

To identify novel catalysts, Brennen et al. (1992, Proc. Natl. Acad. Sci., USA, 89:5381-5383) constructed a heterogenous pool of macromolecules and used an in vitro selection process to isolate molecules that catalyze the desired reaction. A variation of this approach has been used by Gold et al. (U.S. patent no. 5,475,096). This method, known as Systematic Evolution of Ligands by Exponential enrichment (SELEX), identifies nucleic acids that have the ability to form specific, non-covalent interactions with a variety of target molecules. A related patent (U.S. patent no. 5,990,142) is based on

the SELEX method, but can potentially identify modified and non-modified RNA molecules that can catalyze covalent bond formation with a target. Recently, a similar approach was used to identify catalytic RNA molecules having phosphodiesterase, amidase activity (U.S. patent no. 6.063,566 to Joyce).

Additionally, studies have identified RNA molecules that can catalyze aminoacyl-RNA bonds on their own (2')3'-termini. (Illangakekare et al., 1995 Science 267:643-647), or where an RNA molecule can transfer an amino acid from one RNA molecule to another (Lohse et al., 1996, Nature 381:442-444).

However, there has been no demonstration heretofore of catalytic tRNA-like molecules that can cause aminoacylation of RNA molecules which are physiologically significant in modern protein translation processes.

SUMMARY OF THE INVENTION

The present invention provides catalytic RNAs with *cis*-aminoacylation activity. The catalytic RNAs comprise a tRNA-like domain and a ribozyme domain. The ribozyme domain has the catalytic activity and also confers amino acid specificity for aminoacylation. Thus, these catalytic RNAs have the ability to selectively aminoacylate their own 3'-termini with specific amino acids (termed herein as cis-aminoacylating RNA molecules). These catalytic RNAs can be used to aminoacylate tRNA-like sequences that are not naturally aminoacylated.

The present invention also provides catalytic RNA molecules that can aminoacylate tRNA-like molecules in trans (termed herein as trans-aminoacylating RNA molecules). The *trans*-aminoacylating RNAs correspond to the ribozyme domain of the *cis*-aminoacylating RNAs. These catalytic RNA molecules can be used to

aminoacylate tRNA-like molecules with desired natural or non-natural amino acid in *trans*.

The present invention also provides a method of constructing self-aminoacylating RNA molecules. This method comprises the steps of attaching a sequence to the 5' end of a tRNA-like molecule, said sequence comprising a ribozyme sequence. This method can be used to generate catalytic RNAs that can catalyze the aminoacylation of their own 3' ends. These catalytic molecules can be cleaved by RNase P to produce a different species of catalytic RNA molecules which have the ability to aminoacylate tRNA-like molecules in *trans*.

The present invention also provides a method for the identification of RNA sequences having aminoacylation activity. The method comprises the steps of providing a pool of RNA sequences having a tRNA-like domain and a ribozyme domain, contacting the RNA sequences of the pool with the desired natural or non-natural amino acid, partitioning the aminoacylated RNA molecules from the non aminoacylated molecules, amplifying and sequencing the aminoacylated RNA molecules.

The present invention also provides a method for aminoacylating the self-aminoacylating catalytic molecules provided herein. The method comprises the steps of providing RNA molecules having the catalytic activity of self aminoacylating, contacting the RNA molecules with the desired natural or non-natural amino acid, and isolating the aminoacyated RNA molecules.

The present invention also provides a method for aminoacylating tRNA-like molecules. The method comprises the steps of providing a catalytic RNA molecule having a trans-aminoacylating activity, contacting the RNA molecule with a tRNA-like molecule

and the desired amino acid, and isolating the aminoacylated RNA molecules.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic representation of a catalytic RNA with self-aminoacylation activity (left) and a catalytic RNA with trans-aminoacylation activity (right). The amino acid substrate (the amino acid side chain and leaving group are shown with aa and X, respectively) binds to the 5'-leader ribozyme domain, and the nucleophilic attack of the tRNA 3'-hydroxyl (indicated by a curved arrow) is accelerated. The cleavage site of RNase P RNA is shown by the straight arrow.

Figure 1B is a representation of the Secondary structure of otRNA.

Figure 1C is a representation of the chemical structure of substrates cyanomethyl ester (CME), adenosine monophosphate (AMP) (adenylate), and thioester (TE), respectively.

Figure 2 is a representation of an autoradiogram showing self-aminoacylation activity as a function of selection cycle. a, Biotin-Phe-RNA complexed with SAv; b, RNA pool.

Figure 3 is a representation of the sequence alignment of active clones isolated from round 17 RNA. For the alignment of the tRNA domain, the wild-type otRNA is shown together with the selected rtRNA sequences. Consensus deletions and mutations appearing in the tRNA domain are highlighted by boxes. The abbreviations for tRNA loops are: AC, anticodon; V, variable; T, T C.

Figure 4A is a representation of the self-aminoacylation activity and amino acid specificity of pre-24: a, Biotin-aminoacyl-pre-24 complexed with SAv; b, pre-24; c, Biotin-Phe-otRNA complexed with SAv; d, otRNA.

Figure 4B is a representation of comparison of self-aminoacylation activity of pre-24 using three distinct esters. a, Biotin-Phe-pre-24 complexed with SAv; b, pre-24; c, Biotin-Phe-otRNA complexed with SAv; d, otRNA. Reactions were performed in the presence of 0.5 μM pre24 and 5 mM Phe-CME (lane 1), 5 mM Phe-AMP (lane 2), or 10 mM Phe-TE (lane 3) at 25°C (lanes 1 and 3) or 0°C (lane 2) for 30 min.

Figure 4C is a representation of comparison of self-aminoacylation activity of pre-24 and its mutants containing different degrees of mutation and deletion in the tRNA domain. The wild-type pre-24 (lane 1) and pre-24^{otRNA} (lane 5) contain the rtRNA and otRNA sequences of the tRNA domain, respectively.

Figure 5A is a representation of the trans-aminoacylation activity of the 5'-leader ribozyme. Cleavage of pre-24 otRNA by RNase P RNA. a, pre-24 otRIIA ; b, 5' leader segment; c, otRNA.

Figure 5B is a representation of an autoradiogram showing the time course of 5'-leader ribozyme-catalyzed aminoacylation of otRNA. a, Biotin-Phe-otRNA complexed with SAv; b, otRNA. The RNase P-digested RNA fragments of pre-24^{otRNA} were used for aminoacylation (k.b. = 1.0 $x10^{-3}min^{-1}$) .

Figure 5C is a representation of the time course of 5'-leader ribozyme-catalyzed aminoacylation of a minihelix RNA. a, Biotin-Phe-minihelix RNA complexed with SAv; b, minihelix RNA (SEQ ID NO:19) (consisting of the acceptor- T stem-loop region of otRNA).

Figure 6 shows aminoacylation states before and after mild base hydrolysis of aminoacyl-pre-24 with potassium carbonate. Lane 1-3: same as lanes 1-3 in Fig. 4A. Lane 4: Biotin-Phe-pre-24 RNA (same as RNA in lane 1) was treated with 50 mM K_2CO_3 for 15 min at 37°C. Lane 5: RNA recovered from lane 4 was used for aminoacylation under the same conditions as lane 1.

Figure 7 shows aminoacylation of pre-24 and otRNA in the presence of Phe-AMP and Phe-TE. Lane 1: Aminoacylation of pre-24 in the presence of 5mM Phe-AMP followed by biotinylation. Lane 2: Aminoacylation only. Lane 3: Aminoacylation of otRNA with 5mM Phe-AMP followed by biotiylation. Lane 4: Aminoacylation of pre-24 in the presence of 10mM Phe-TE followed by biotinylation. Lane 5: Aminoacylation only. Lane 6: Biotinylation only. Lane 7: Aminoacylation of otRNA with 10mM Ohe-TE followe by biotinylation.

Figure 8 shows ribozyme-catalyzed aminoacylation on tRNA variants *in trans*. Reactions were carried out for 3 hours. The v1 and v3 (lanes 2 and 3) are the fragment of the tRNA domain described in Figure 4C.

DETAILED DESCRIPTION OF THE INVENTION

The phrase "tRNA-like molecules" or "tRNA-like domains" or "tRNA-like sequences" as used herein for the purposes of specification and claims, means RNA molecules that have sequences consistent with the formation of a cloverleaf structure typically associated with tRNAs. An example of a tRNA-like molecule is otRNA (SEQ ID NO:18). Other t-RNA-like molecules are the tRNA domains of SEQ ID NO:5-15 i.e., nucleotides 86-146 of SEQ ID NO:5, nucleotides 90-151 of SEQ ID NO:6, nucleotides 90-150 of SEQ ID NO:7, nucleotides 89-150 of SEQ ID NO:8, nucleotides 90-150 of SEQ ID NO:9, nucleotides 89-149 of SEQ ID NO:10, nucleotides 89-149 of SEQ ID NO:11, nucleotides 89-149 of SEQ ID NO:12, nucleotides 89-149 of SEQ ID NO:13, nucleotides 90-150 of SEQ ID NO:14, and nucleotides 89-148 of SEQ ID NO:15. Still other tRNA-like molecules are SEQ ID NO:20-22.

The phrase "ribozyme" as used herein for the purposes of specification and claims, means an RNA molecule that is capable of catalyzing a chemical reaction.

The phrase "natural amino acid" refers to any amino acid among the twenty amino acids that are normally aminoacylated onto tRNAs in living cells. Such amino acids are alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, methionine, glycine, serine, threonine, tyrosine, cysteine, glutamine, asparagine, lysine, arginine, histidine, aspartic acid, and glutamic acid. Consequently, the phrase "non-natural amino acids" means any amino acid other than the natural amino acids or modification of a natural amino acid.

The term "cis" means within the same molecule. The term "trans" means on or to a different molecule.

The term "5'-leader domain" or "catatlytic domain" or "5'-leader ribozyme" means the 5' region of the catalytic molecules having *cis*-aminoacylating activity. Examples of such 5'-leader domains are nucleotides 1-85 of SEQ ID NO:5, nucleotides 1-89 of SEQ ID NO:6, nucleotides 1-89 of SEQ ID NO:7, nucleotides 1-88 of SEQ ID NO:8, nucleotides 89 of SEQ ID NO:9, nucleotides 1-88 of SEQ ID NO:10, nucleotides 1-88 of SEQ ID NO:11, nucleotides 1-88 of SEQ ID NO:12, nucleotides 1-88 of SEQ ID NO:13, nucleotides 1-89 of SEQ ID NO:14 and nucleotide 1-88 of SEQ ID NO:15.

The present invention is directed to catalytic RNA molecules that can catalyze aminoacylation reactions. The catalytic RNA molecule having self-aminoacylating activity has two domains, a catalytic domain and an aminoacyl acceptor domain. The catalytic domain contains a sequence which has ribozymal activity. This domain alos confers amino acid specifity.

To construct the catalytic RNA molecules having self aminoacylation activity, a selection process is used on a randomly synthesized RNA pool. By attaching the RNA molecules of this pool to the 5' end of a tRNA-like molecule and contacting the complex with a substrate molecule (natural or non-natural amino acid),

self-aminoacylating RNA molecules are identified. These molecules can then be selectively amplified. The self-aminoacylating nature of these molecules can be confirmed by standard assays such as mobility-gel-shift assays. To facilitate the isolation of the active aminoacylated RNA species, a biotin tag may be attached to the target molecule. Alternatively, a water soluble form of biotin may be used to label the aminoacylated RNAs subsequent to generation of the aminoacylated RNA species. These biotin labeled species can be isolated by using strepavidin coated agarose. Repeated rounds of selective amplification yield pure species of the catalytic RNA with self aminoacylating activity. A round of selection comprises in part reverse - transcribing RNAs collected from the SAv bound portion of the reaction in order to produce cDNAs. The cDNAs are subjected to the polymerase chain reaction (PCR) followed by transcription of the PCR products to generate RNAs used in the next round of selection. The RNAs obtained from the final selection round are subjected to mobility gel-shift assays to demonstrate self-aminoacylation and trans-aminoacylation activity.

The catalytic molecules of the present invention can also aminoacylate RNA species in trans. To produce such catalytic molecules, the aminoaccyl acceptor domain (the tRNA-like domain) can be cleaved from the catalytic domain by bacterial RNase P digestion. The catalytic domain is able to aminoacylate the aminoacyl acceptor domain or a tRNA-like molecule in *trans*. The catalytic molecules can also be synthesized by *in vitro* run-off transcription catalyzed by an appropriate RNA polymerase from the corresponding DNA templates by methods known to those skilled in the art.

The aminoacyl acceptor domain is a tRNA-like molecule. A suitable example of a tRNA-like molecule is an artificial orthogonal suppressor tRNA (otRNA see Fig.

IB). Alternatively, an amber supressor tRNA derived or isolated from different species, such as human, can be used for this purpose. This tRNA sequence is derived from an amber suppressor $tRNA^{Gln2}$ but is not recognized by bacterial ARSs. The otRNA can be attached to unique catalytic domains that confer aminoacylation activity and amino acid specificity.

Once sequences known to possess aminoacylating activity are isolated, they can be used in a scaffold-based approach to isolating additional aminoacylating sequences. The pre-24^{phe} sequence region (except for the 5'-primer sequence) in DNA form is randomly mutagenized such that each base has a certain percentage chance of being non-wild-type. This DNA pool is then annealed with the anistense DNA of the otRNA, and the annealed pool is extended to full-length duplex DNA. New copies of the templates are generated by PCR, these are transcribed and subject to selection as previously described.

Taking advantage of the catalytic scaffold of the pre-24^{Phe} allows screening of a focused sequence space in which most sequences can maintain the secondary or even tertiary structures of the pre-24^{Phe}. Despite this narrowed focus, the scaffold pool has enough random mutations to give the desired alteration of amino acid specificity dependant upon the amino-acid chosen to be the amino acid component of the substrate. Therefore, the likelihood of encountering active sequences can become higher than the selection using a completely random pool. For the scaffolding pool, it is unnecessary to deal with a high complexity pool because fewer nucleotides are randomized (depending upon the mutation rate, its complexity can be two or three orders of magnitude lower). Therefore, the selection is much less labor-intensive and the outcome can be obtained more rapidly.

These and other embodiments will become more clear from the examples described below, which are to be construed as illustrative and not limiting.

EXAMPLE 1

This embodiment describes the construction of a pool of RNA molecules for screening of aminoacylation activity. In an illustration of this embodiment, randomized sequences were generated, attached to a tRNA-like molecule. Thus, a random pool of 70 nucleotides was attached to the 5' end of a tRNA-like molecule (otRNA) as follows.

Pool construction: Four synthetic oligonucleotides were used for the pool construction: the random pool DNA template (5'-GGATCGTCAGTGCATTGAGA-N70-GGTGGTATCCCCAAGGGGTA-3')(SEQ ID No. 1), the DNA template complementary to the orthogonal tRNA (otRNA)(SEQ ID No. 2), the 5'-primer containing T7 promoter sequence (SEQ ID No. 3) , and 3'-primer (SEQ ID No. 4) A large-scale Taq DNA polymerase- extension of the DNA templates was performed under thermocylcing conditions (95°C for 10 min, 55°C for 10 min, and 72°C for 10 min). The full-length product was then amplified by seven cycles of large-scale PCR in the presence of the 5'- and 3'-primers. Four equivalents of the pool DNA with an approximately 10^{15} complexity were transcribed by T7 RNA polymerase in the presence of a-[^{32}P]-UTP, and purified by 6% polyacrylamide gel electrophoresis (PAGE).

EXAMPLE 2

This embodiment describes the selection of active sequences from the RNA molecules constructed in Example 1. As illustrations, the following substrates were used to select aminoacylating species of RNA.

Substrates

N-biotinyl-L-aminoacyl-cyanomethyl esters (Biotin-aa-CME) were chosen as the aminoacyl donor substrates because the CME group has a balance of activation and hydrolytic stability. Furthermore, CME has no hydrogen-bonding functionalities, which helps to ensure that the primary interaction with RNA will occur through the amino acid side chain of the substrate.

Other substrates included Pheylalanyl adneylate (Phe-AMP) and pheylalanyl thioester (Phe-TE). These substrates were also chosen for reasons described above and to demonstrate specificity to the amino acid rather than other components of the substrate. The biotin tag facilitates the isolation of active, i.e. aminoacylated, sequences on immobilized streptavidin (SAv) agarose via an interaction between the biotin moiety of the substrate and the SAv. N-biotinyl-L-phenylalanyl-cyanomethyl esters (Biotin-Phe-CME) and Boc-Phe-CME (Boc is tert-butoxy carbonyl) were synthesized essentially by the procedure as previously described (Suga, et al. *J. Am. Chem. Soc.* 120: 1151-1156 1998). Synthesis of Phe-CME was carried out as follows: 9:1 TFA/anisole solution (500 mL) was added to Boc-Phe-CME (385 mg, 1.26 mmol) under argon atmosphere and the mixture was stirred at room temperature for 30 min. The solvent was removed in vacuo and ca. 4 M hydrogen chloride in dioxane (4 mL) was added to the residue. The solution was concentrated in vacuo, and the addition of anhydrous ether to the residue yielded precipitate. The precipitate was dissolved in a minimal amount of MeOH, and to this solution anhydrous ether was added to reprecipitate the product. Purity of the product was determined using nuclear magnetic resonance (NMR).

Synthesis of phenylalanyl adenylate (Phe-AMP) was carried out essentially as described previously (Berget al. *Bio. Chem.* 253: 608-611, 1958). ^{32}P NMR analysis of the product indicated that the purity of Phe-AMP was

approximately 50% and the remaining side-product was unreacted AMP. The product was dissolved in water, and used for aminoacylation without further purification.

Synthesis of phenylalanyl thioester (Phe-TE) was as follows: N,N-bis[2-oxo-3-oxozolidiyllphosphordiamitic chloride (238 mg, 0.94 mmol) was added to a solution of Boc-Phe (307 mg, 1.16 mmol) and triethylamine (175 mL, 2.26 mmol) in CH2C12 (10 mL) . To this mixture, ethyl 2-mercapto acetate (100 mL, 0.91 mmol) was slowly added, and the reaction mixture was then stirred vigorously for 5 h at room temperature. The reaction was quenched by the addition of 20% $NaHCO_3$ aqueous solution. After standard aqueous work-up, Boc-Phe-TE was isolated by column chromatography. 9:1 TFA/anisole solution (500mL) was added to Boc-Phe-TE (200 mg, 0.30 mmol) under argon atmosphere, and the mixture was stirred at room temperature for 30 min. The solvent was removed in vacuo and ca. 4M hydrogen chloride in dioxane (4 mL) was added to form the hydrochloride salt. The solution was concentrated in vacuo, and the residue was dissolved in ether. The addition of petroleum ether to this solution resulted in the formation of precipitate, which was rinsed with ether and filtered to yield Phe-TE.

Selection of Active Sequences

Selection reactions were carried out under the following conditions: A mixture of 10 μM (first round only) or 1 μM RNA pool, 1 mM Biotin-Phe-CME, in an EK buffer (50 mM EPPS, 500 mM KC1, pH 7.5), 100 MM $MgCl_2$, and ethanol (25% of the total volume) The pool RNA was preincubated in the EK buffer, heated at 95°C for 5 min, and cooled to 25°C over 5 min. $MgCl_2$ was then added followed by a 5 min equilibration. The reaction was initiated by the addition of the substrate solution in ethanol, and incubated for 3 h at 25 °C (30 min in the 15-17th rounds). The reaction was stopped by adding 2 volumes of cold ethanol, and the RNA was ethanol-precipitated twice. The RNA pellet was dissolved into

EKE buffer (50 mM EPPS, 500 mM KC1, 5 mM EDTA, pH 7.5), then incubated with 200 μL (1 mL for the first round) of streptavidin agarose for 30 min at room temperature. Unbound RNAs were eluted with 20-resin volumes of the EKE buffer, 40-resin volumes of 4 M urea, then 10-resin volumes of water. The resin-bound RNAs were eluted by heating at 95°C for 10 min in the presence of 10 mM biotin at pH 7. The collected RNAs were reverse-transcribed using 100 units M-MLV reverse transcriptase (Promega™) in the presence of 1 μM 3'primer (SEQ ID No. 4), 125 μM dNTPs, 50 mM Tris-HC1, 75 mM KC1, 3 MM $MgC1_2$, 10 mM DTT, pH 8.3 at 42°C for 1 h (for 1-14 rounds) or 10 min (for 15-17 rounds). The cDNAs were subjected to PCR followed by transcription under standard conditions.

Fifteen rounds of selective amplification of self-aminoacylating RNA molecules in the pool yielded an enrichment of active sequences, which was confirmed by a SAv-dependent mobility-gel-shift assays (Figure 2, lanes 1-3). Approximately 10% of the total input RNA molecules from round 15 showed aminoacylation after 3 h (lane 3). Two rounds of selection with shorter incubation times were employed in order to further enhance the activity in the pool (lanes 4-6). The absence of SAv or substrate resulted in loss of the retarded band (lanes 7,8), indicating that self-aminoacylation of active RNAs with the Biotin-Phe group is occurring. Periodate oxidation of the 3' terminal diol or deletion of the 3' adenosine resulted in near complete inhibition of aminoacylation (lanes 9,10), strongly suggesting that the 3'terminal hydroxyl group is the aminoacylation site.

Thirty-six individual clones form the round 17 pool were were screened for self-aminoacylation (self aminoacylation assay described in Example B), and 11 clones exhibited appreciable activity (SEQ ID NO:5-15). Alignment of their sequences revealed an approximately 95% identity in the 5' leader domains (Fig. 3, top). A

representative clone, termed as the pre-24 or rtRNA (SEQ ID NO:9), was selected for confirmation of the self-aminoacylation activity.

EXAMPLE 3

The self-aminoacylation activity of pre-24 in the presence of Biotin-Phe-CME was assayed under conditions similar to those of the selection except that 12.5 mM KCl and 5% ethanol were used. At each time point, an aliquot of the reaction was ethanol-precipitated twice, and the pellet was dissolved into 7 μL a MEUS buffer (25 mM MOPS, 5 mM EDTA, 8 M Urea, 10 μM streptavidin, pH 6.5). The reactions were carried out in the presence of 1 μM RNA and 1 mM Biotin-Phe-CME (Fig. 4, lanes 1, 2, 4, and 5) or 1 mM Biotin-aa-CME (lanes 6-10) or in the absence of substrate (lane 3). For periodate oxidation (lane 4), pre-24 was treated with 10 mM NaOH at 0 ^{o}C for 1 h and ethanol-precipitated prior to the aminoacylation reaction. The reactions were incubated for 30 min (lanes 1-4) or 2 h (lanes 5-10). The resulting solution was analyzed by 10% PAGE in a cold room in order to keep the gel temperature below 20 ^{o}C (Fig. 4). A plot of initial rates vs. substrate concentration revealed Michaelis-Menten behavior with kinetic parameters of k_{cat} =0.10 + 0.01 min^{-1} and Km = 6.3 ± 1.2 mM, while the solubility limit of substrate constrains its actual working concentration to below 5 mM. The background rate was determined by incubating otRNA with 1 mM Biotin-Phe-CME for 3 h in the same reaction buffer as above, giving a yield of 0.01% of the aminoacyl-otRNA (lane 11). The background rate is thus estimated to be an approximately 5.5 X $10^{-7}Min^{-1}$. The observed rate acceleration by ribozyme is approximately 10^{5}-fold greater than background.

The self-aminoacylation activity was confirmed by the SAv-dependent mobility-gel-shift assay (Fig. 4A, lanes 1-3). Periodate oxidation completely eliminated

activity, strongly suggesting that the aminoacylation site is the 3'-end (lane 4). Mild base hydrolysis of aminoacyl-pre-24 with potassium carbonate resulted in loss of the retarded band, i.e. Biotin-Phe was hydrolyzed from pre-24. When re-exposed to the aminoacyl substrate this deacylated pre-24 still showed full self-aminoacylation activity (see Fig. 6). This suggests that a 3' or 2'-ester bond is the only plausible linkage of the aminoacyl-pre-24.

The amino acid specificity of catalytic RNAs was investigated by using five distinct Biotin-aminoacyl-CMEs (Fig. 4, lanes 6-10). For substrates of Phe-CME, Phe-AMP and Phe-TE, self-aminoacylation reactions were carried out with the same procedures as those described as above, except that the aminoacyl-RNA pellet was resuspended in an acidic EPPS buffer (0.3 M, pH 5.5). 0.3 M EPPS-KOH was then added to this solution, which brought the pH to 8.0.

Reaction rates for these amino acids were drastically reduced compared with phenylalanine, indicating that the ribozyme domaine has a remarkable specificity toward Biotin-Phe-CME.

To further define the primary recognition element in the substrate, three synthetic phenylalanyl esters (Fig. 1C) were tested for activity (Fig. 4B) (16). Reactions were performed in the presence of 0.5 μM pre24 catalytic RNA and 5 mM Phe-CME (lane 1), 5 mM Phe-AMP (lane 2), or 10 mM Phe-TE (lane 3) at 25 ^{o}C (lanes 1 & 3) or 0 ^{o}C (lane 2) for 30 min. The absence of biotinylation (lane 4) or substrate (lane 5) for Phe-CME resulted in loss of the retarded band, indicating that the aminoacylation is necessary for the retarded band. The background aminoacylation was monitored using otRNA in the presence of 5 mM Phe-CME (lane 6). The same control experiments for Phe-AMP and Phe-TE were also performed (see figure 7). Omission of biotin from the a-amino group, i.e. Phe-CME, gave almost the same catalytic rate

as that observed for Biotin-Phe-CME (Fig. 4B, lane 1) when the concentration of Phe-CME increased by 5-fold. This illustrates that the biotinyl group, presumably the amide functionality on the a-amino group, interacts with ribozyme, but it is not an essential element for substrate recognition. The ribozyme also accommodated the adenylate (Phe-AMP) and a thioester (Phe-TE) in place of the CME leaving group (lanes 2&3). This demonstrated that the critical recognition element of the substrate is the phenylalanyl side chain, not the leaving group.

EXAMPLE 4

This embodiment demonstrates the preparation and trans-aminoacylating activity of the catalytic RNA molecules. The pre-24 catalytic RNA from Example 1 and 2 was used in this illustration. First, pre-24 otRNA was subjected to RNase P scission (Fig. 5A) to liberate the 5'-catalytic domain from the 3'-tRNA domain. A [^{32}P]-body-labeled pre-24^{otRNA} was treated with RNase P RNA for 2 h, resulting in the cleavage of approximately 23% of pre-24^{otRNA} (lane 1). The absence of RNase P yielded no cleaved product (lane 2). The marker RNAs (5'-leader segment in lane 3 and otRNA in lane 4) were prepared by in vitro transcription using the corresponding DNA segments. E. coli RNase P RNA was in vitro transcribed using a PCR-amplified DNA template from the M1 gene of the pDW27 plasmid as previously described (Ziehler et a. (1996) *Biotechniques* 20, 622-624), then purified on 6% PAGE. The cleavage of pre-24^{otRNA} (1 μM) by the RNase P RNA (1 μM) was carried out in I M NH_4OAc, 50 mM $MgCl_2$ and 0.1 % SDS at 37 ^{o}C (14). After the reaction, the solution was ethanol-precipitated twice and the resulting solution was analyzed by 10% PAGE.

Treatment of pre-24^{otRNA} with E.coli RNase P RNA

produced two fragments of lengths corresponding to the 5'-leader segment and the otRNA (Fig. 5A, lanes 1 and 2, compare with in vitro transcripts of each fragment in lanes 3 and 4). This demonstrates that pre-24^{otRNA} is susceptible to RNase P RNA hydrolysis. Thus, the catalytically active pre-tRNA can be segmented tRNA-like molecule and a 5' leader segment.

Next, it was examined whether the 5'-leader fragment could aminoacylate the otRNA fragment in trans (Fig. IA, right). For the analysis of the trans-aminoacylation activity (Fig. 5B), an unlabeled pre-24^{otRNA} was cleaved, and the individual segments of otRNA and 5'leader domain were purified by 10%; PAGE. The tRNA segment was treated with calf intestinal alkaline phosphatase and then phosphorylated using T4 polynucleotide kinase in the presence of [^{32}P]-a-ATP. The 5'-leader and otRNA fragments generated by RNase P RNA action on pre-24^{otRNA} were treated with Biotin-Phe-CME (Fig. 5B). The 5'-leader fragment transaminoacylated otRNA at a rate similar to the cis reaction of pre-24^{otRNA}. The in vitro transcribed 5'-leader fragment also exhibited trans-activity similar to that observed above. Thus, the RNase P RNA-digested 5'leader fragment can independently fold into its functional structure, and act as a trans-acting aminoacylation enzyme.

The substrate properties of a minihelix RNA consisting of the acceptor- T stem-loop region of otRNA (Fig. 5C) were also tested. The 5'-leader ribozyme and minihelix RNA were in vitro transcribed, then purified on PAGE. Prior to the reaction, each of the RNAs (4 μM of 5' leader ribozyme and 3 μM of minihelix RNA) were folded independently. The remaining procedures were the same as described in the method section. The minihelix RNA was still aminoacylated by 5'-leader ribozyme, indicating that the anticodon loop is not essential for activity. An approximately 4-fold reduction of the observed rate as compared to otRNA, however, suggests

that 5'-leader ribozyme interacts with additional elements present in the full-length of otRNA.

Additionally, the 5'-leader fragment *trans*-aminoacylated otRNA at a rate similar to the *cis* reaction of pre-24^{otRNA} . The *in vitro* transcribed 5'-leader fragment also exhibited *trans*-activity similar to that observed above. In addition, this fragment showed activity toward rtRNA and its variants at similar rates to the respective *cis* reactions (Fig. 8).

In conclusion, the present invention provides modifed tRNAs with the ability to catalyze the aminoacylation of their own 3' ends. The 5'-leader sequences may exist independently of the 3'-tRNA domains and still retain their catalytic property.

EXAMPLE 5

A scaffolding strategy was used for the selection of Leu-specific ribozymes using a 15%-mutagenized scaffold pool. The 15%-mutagenized scaffold RNA pool was synthesized as follows: Each nucleotide position of ribozyme, except for the 5'-primer region and the 5'-overlapped region of tRNA, is mutagenized on the corresponding DNA template by using an automated DNA synthesizer. Prior to the synthesis, each phosphoramidite base was mixed with the other three bases with the reactivity ratio of 85:5:5:5. The DNA template was synthesized according to the ribozyme sequence. The deprotection and purification of oligonucleotide was employed as standard methods. The DNA was amplified by the same method as described in Example 1, except that the PCR was carried out in a 200 μL scale. In vitro transcription of this DNA template followed by purification on PAGE gave the 15%-mutagenized scaffold RNA pool. After only five rounds of selection, active sequences appeared in the pool (whereas selection of pre-24^{Phe} from a completely random

pool required 15 rounds). It should be noted that the observed activity toward orthogonal tRNA (otRNA) is even higher than that of the original pre-24^{Phe}, indicating that this strategy is effective not only for switching the specificity but also for optimizing activity toward otRNA.

By using this approach, leucine specific cis aminoacylating RNAs were obtained. Two examples are presented as SEQ ID NO:17 and SEQ ID NO;18.

Although preferred embodiments of the present invention have been described and illustrated herein, the present invention is not limited to such preferred embodiments. Those skilled in the art will appreciate that various changes may be made without departing from the spirit of the invention.

What is claimed is:

1. A nucleic acid molecule comprising a polynucleotide selected from the group consisting of:

a. RNA having a sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15, and complementary sequences thereof;

b. DNA having the sequence of a) wherein t is substituted for u.

2. The nucleic acid molecule of claim 1, wherein the RNA sequence has a sequence of SEQ ID NO:9.

3. A nucleic acid molecule comprising a polynucleotide selected from the group consisting of:

a) RNA having a sequence of nucleotides 1-85 of SEQ ID NO:5, nucleotides 1-89 of SEQ ID NO:6, nucleotides 1-89 of SEQ ID NO:7, nucleotides 1-88 of SEQ ID NO:8, nucleotides 89 of SEQ ID NO:9, nucleotides 1-88 of SEQ ID NO:10, nucleotides 1-88 of SEQ ID NO:11, nucleotides 1-88 of SEQ ID NO:12, nucleotides 1-88 of SEQ ID NO:13, nucleotides 1-89 of SEQ ID NO:14, nucleotide 1-88 of SEQ ID NO:15, and complementary sequences thereof; and

b) DNA having the sequence of a) wherein t is substituted for u.

3. A nucleic acid molecule comprising the polynucleotide selected from the group consisting of:

a) RNA having a sequence of nucleotides 86-146 of SEQ ID NO:5, nucleotides 90-151 of SEQ ID NO:6, nucleotides 90-150 of SEQ ID NO:7, nucleotides 89-150 of SEQ ID NO:8, nucleotides 90-150 of SEQ ID NO:9, nucleotides 89-149 of SEQ ID NO:10, nucleotides 89-149 of SEQ ID NO:11, nucleotides 89-149 of SEQ ID NO:12, nucleotides 89-149 of SEQ ID NO:13, nucleotides 90-150

of SEQ ID NO:14, nucleotides 89-148 of SEQ ID NO:15, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, complementary sequences thereof; and

b) DNA having a sequence of a) wherein t is substituted for u.

4. The nucleic acid molecule of claim 3, wherein the RNA has the sequence of nucleotides 90-150 of SEQ ID NO:9

5. A method for constructing cis-aminoacylating catalytic RNA molecules comprising the steps of providing tRNA-like molecules and attaching a ribozyme sequence to the 5' end of the tRNA like molecule, said ribozyme sequence being selected from the group consisting of nucleotides 1-85 of SEQ ID NO:5, nucleotides 1-89 of SEQ ID NO:6, nucleotides 1-89 of SEQ ID NO:7, nucleotides 1-88 of SEQ ID NO:8, nucleotides 89 of SEQ ID NO:9, nucleotides 1-88 of SEQ ID NO:10, nucleotides 1-88 of SEQ ID NO:11, nucleotides 1-88 of SEQ ID NO:12, nucleotides 1-88 of SEQ ID NO:13, nucleotides 1-89 of SEQ ID NO:14 and nucleotide 1-88 of SEQ ID NO:15.

6. A method of identifying cis-aminoacylating catalytic RNA molecules comprising the steps of:

a. providing a tRNA-like molecule;

b. providing a ribozyme domain molecule;

c. attaching the ribozyme domain molecule to the 5' end of the tRNA-like molecule to obtain a pool of ribozyme-tRNA molecules;

d. contacting the ribozyme-tRNA molecules with an amino acid substrate;

e. partitioning the aminoacylated ribozyme-tRNA molecules from the remainder of the ribozyme-tRNA molecules to obtain cis-aminoacylating catalytic RNA molecules.

7. The method of claim 6, wherein the tRNA-like molecule has a sequence selected from the group consisting of nucleotides 86-146 of SEQ ID NO:5, nucleotides 90-151 of SEQ ID NO:6, nucleotides 90-150 of SEQ ID NO:7, nucleotides 89-150 of SEQ ID NO:8, nucleotides 90-150 of SEQ ID NO:9, nucleotides 89-149 of SEQ ID NO:10, nucleotides 89-149 of SEQ ID NO:11, nucleotides 89-149 of SEQ ID NO:12, nucleotides 89-149 of SEQ ID NO:13, nucleotides 90-150 of SEQ ID NO:14, nucleotides 89-148 of SEQ ID NO:15, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22 and complementary sequences thereof.

8. The method of claim 6, wherein the ribozyme domain molecule has a sequence selected from the group consisting of nucleotides 1-85 of SEQ ID NO:5, nucleotides 1-89 of SEQ ID NO:6, nucleotides 1-89 of SEQ ID NO:7, nucleotides 1-88 of SEQ ID NO:8, nucleotides 89 of SEQ ID NO:9, nucleotides 1-88 of SEQ ID NO:10, nucleotides 1-88 of SEQ ID NO:11, nucleotides 1-88 of SEQ ID NO:12, nucleotides 1-88 of SEQ ID NO:13, nucleotides 1-89 of SEQ ID NO:14 and nucleotide 1-88 of SEQ ID NO:15.

9. A method of obtaining a *trans*-aminoacylating catalytic RNA molecule comprising the steps of:

a. providing a RNA molecule having a sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15,

b. cleaving the RNA molecule with RNase P to obtain the trans-aminoacylating catalytic RNA molecule and an aminoacyl acceptor domain;

10. A method of cis-aminoacylating a catalytic RNA molecule comprising the steps of:

a. providing catalytic RNA molecules having an cis-aminoacylation activity;

b. contacting the RNA molecules with the desired natural or non-natural amino acid; and

c. isolating aminoacylated RNA molecules.

11. A method of trans-aminoacylating a tRNA-like molecule comprising the steps of:

a. providing catalytic RNA molecules having a trans-aminoacylation activity;

b. providing a tRNA-like molecule;

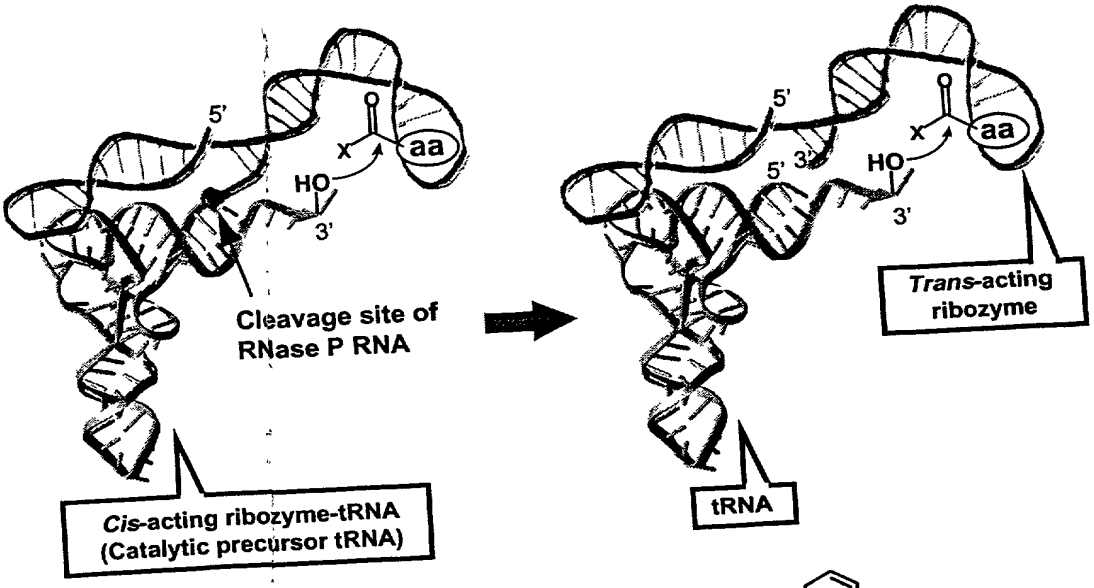
c. contacting the catalytic RNA molecules and the tRNA-like molecules with the desired natural or non-natural amino acids; and

d. isolating aminoacylated tRNA-like molecules.

ABSTRACT

The present invention provides catalytic RNA molecules having cis or trans aminoacylation activity. The catalytic RNA molecules having cis aminoacylation activity comprise a catalytic domain and an aminoacylation domain. The catalytic RNA molecules having trans aminoacylation activity only have the catalytic domain. A method is provided for constructing and screening of these molecules. These molecules are suitable for aminoacylating tRNA-like molecules with specific amino acids.

Figure 1A



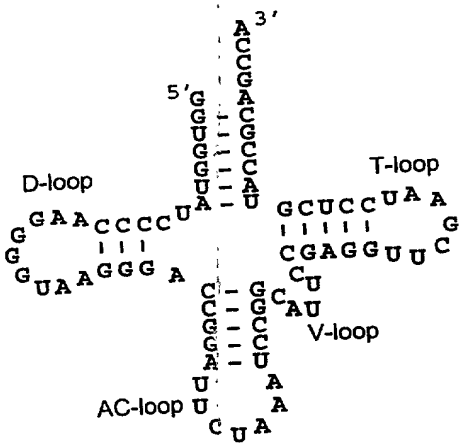


Figure 1B

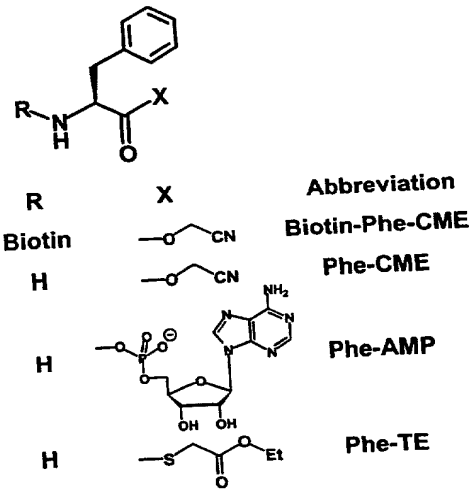


Figure 1C

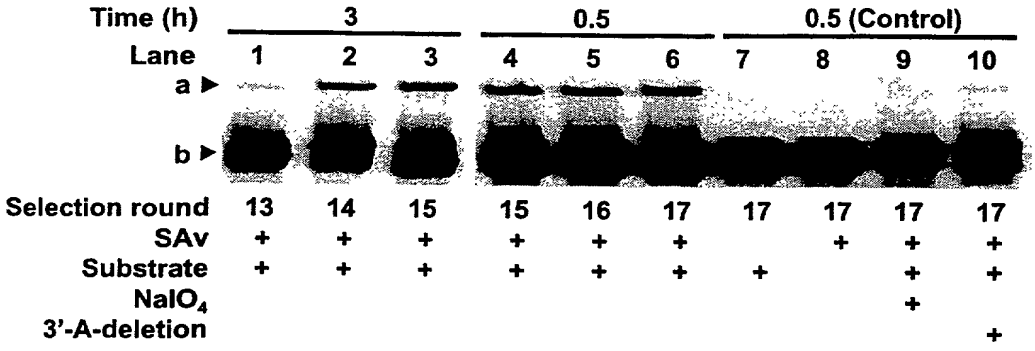

Time (h)
3
0.5
0.5 (Control)
Lane
1 2 3 4 5 6 7 8 9 10
a ▶
b ▶
Selection round
13 14 15 15 16 17 17 17 17 17
SAv
+ + + + + + + + +
Substrate
+ + + + + + + + +
NaIO4
+
3'-A-deletion
+

Fig. 2

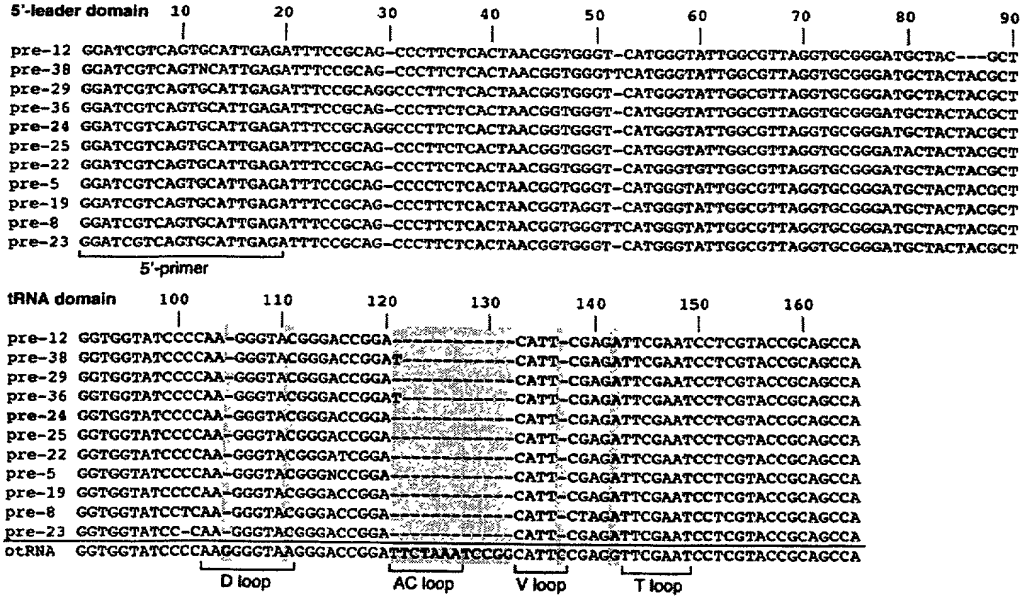

5'-leader domain
10 20 30 40 50 60 70 80 90
pre-12 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTAC---GCT
pre-38 GGATCGTCAGTNCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGTTCATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-29 GGATCGTCAGTGCATTGAGATTTCCGCAGGCCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-36 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-24 GGATCGTCAGTGCATTGAGATTTCCGCAGGCCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-25 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-22 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGT-CATGGGTGTTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-5 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCCTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-19 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTAGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-8 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGTTCATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
pre-23 GGATCGTCAGTGCATTGAGATTTCCGCAG-CCCTTCTCACTAACGGTGGGT-CATGGGTATTGGCGTTAGGTGCGGGATGCTACTACGCT
5'-primer
tRNA domain
100 110 120 130 140 150 160
pre-12 GGTGGTATCCCCAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-38 GGTGGTATCCCCAA-GGGTACGGGACCGGAT---------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-29 GGTGGTATCCCCAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-36 GGTGGTATCCCCAA-GGGTACGGGACCGGAT---------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-24 GGTGGTATCCCCAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-25 GGTGGTATCCCCAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-22 GGTGGTATCCCCAA-GGGTACGGGATCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-5 GGTGGTATCCCCAA-GGGTACGGGNCCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-19 GGTGGTATCCCCAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
pre-8 GGTGGTATCCTCAA-GGGTACGGGACCGGA----------CATT-CTAGATTCGAATCCTCGTACCGCAGCCA
pre-23 GGTGGTATCC-CAA-GGGTACGGGACCGGA----------CATT-CGAGATTCGAATCCTCGTACCGCAGCCA
otRNA GGTGGTATCCCCAAGGGGTAAGGGACCGGATTCTAAATCCGGCATTCCGAGGTTCGAATCCTCGTACCGCAGCCA
D loop
AC loop
V loop
T loop

Fig. 3

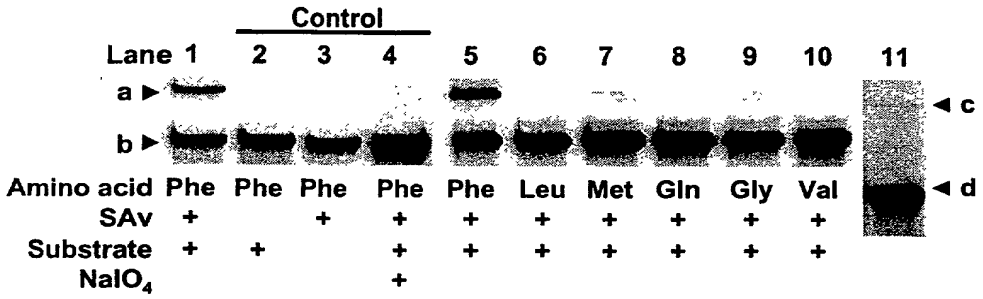


Figure 4A

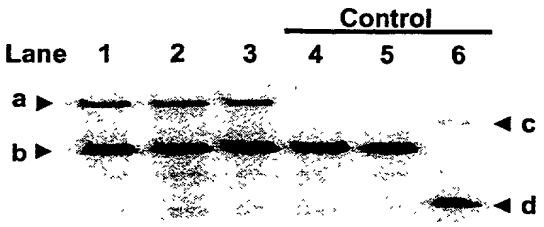


Figure 4B

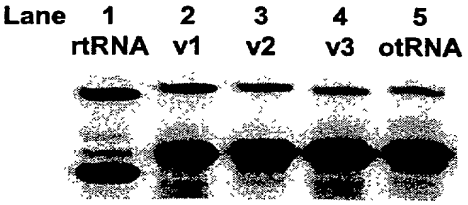


Figure 4C

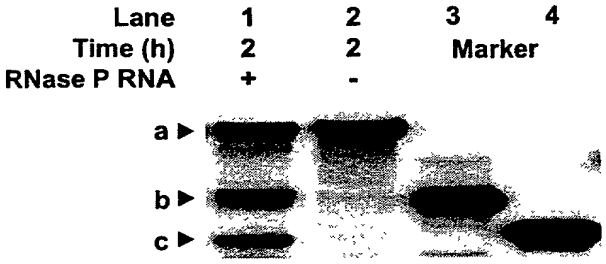


Figure 5A

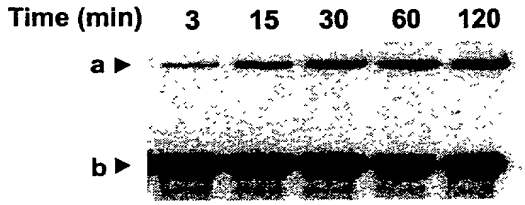


Figure 5B

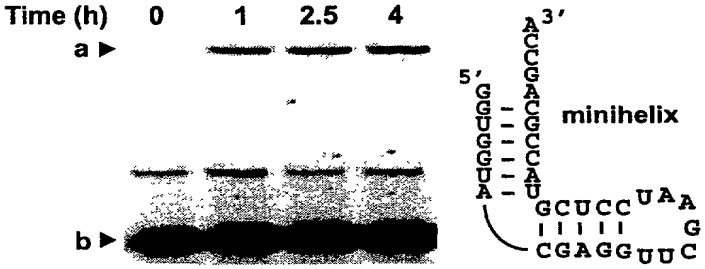


Figure 5C

Figure 6

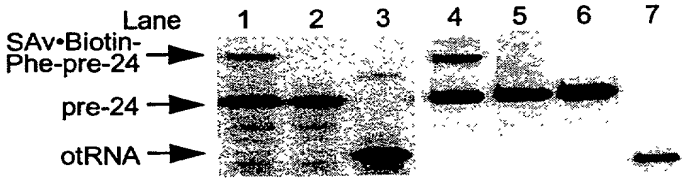


Figure 7

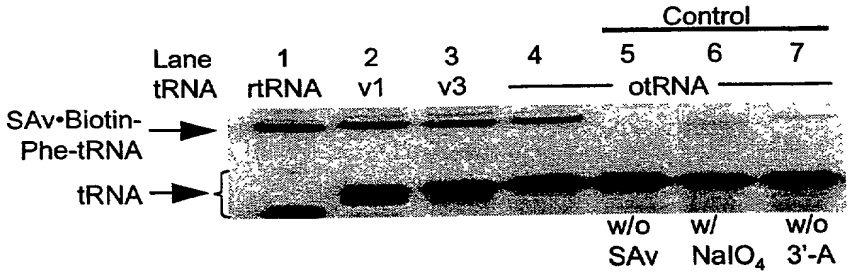


Figure 8

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing **OR** ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

Attorney Docket Number	11520.0222
First Named Inventor	Suga, et al
COMPLETE IF KNOWN	
Application Number	
Filing Date	November 22, 2000
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Catalytic RNAs With Aminoacylation Activity

(Title of the Invention)

The specification of which
☐ is attached hereto
OR
☐ was filed on (MM/DD/YYYY) ☐ as United States Application Number or PCT International Application Number ☐ and was amended on (MM/DD/YYYY) ☐ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application (Numbers)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	**Filing Date (MM/DD/YYYY)**	
60/214,382 60/167,331	06/28/2000 11/24/1999	☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☐ Customer Number
OR
■ Registered practitioner's name/registration number listed below

→ *Place Customer Number Bar Code Label Here*

Name	Registration Number	Name	Registration Number
Ranjana Kadle	40,041	R. Kent Roberts	40,786
John M. DelVecchio	42,475	Michael F. Scalise	34,920
Martin G. Linihan	24,926	Patrick J. Tracy	42,187
Kevin D. McCarthy	35,278	Daniel C. Oliverio	33,435
David L. Principe	39,336	Edwin T. Bean, Jr.	16,639

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto

Direct all correspondence to: ☐ Customer Number or Bar Code Label *OR* ■ Correspondence address below

Name	Ranjana Kadle				
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Address	One M&T Plaza, Suite 2000				
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Country	United States	**Telephone**	(716) 856-4000	**Fax**	(716) 849-0349

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Hiroaki	Suga

Inventor's Signature						**Date**	
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Post Office Address	41 Fenwick Road						
Post Office Address							
City	Williamsville	**State**	New York	**ZIP**	14221	**Country**	US

■ Additional inventors are being named on the _2_ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

DECLARATION	ADDITIONAL INVENTOR(S) **Supplemental Sheet** Page 3 of 4

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
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Inventor's Signature					**Date**		
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City	Williamsville	**State**	New York	**ZIP**	14221	**Country**	US

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle [if any])				Family Name or Surname			
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Inventor's Signature					**Date**		
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Post Office Address							
City	Buffalo	**State**	New York	**ZIP**	14261	**Country**	US

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle [if any])				Family Name or Surname			
Nick				Lee			
Inventor's Signature					**Date**		
Residence: City	Snyder	**State**	New York	**Country**	US	**Citizenship**	US
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Post Office Address							
City	Snyder	**State**	New York	**ZIP**	14226	**Country**	US

DECLARATION	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 4 of 4

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
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Neil				Bonzagni			
Inventor's Signature						**Date**	
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Post Office Address							
City	Buffalo	**State**	New York	**ZIP**	14261	**Country**	US

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature						**Date**	
Residence: City		**State**		**Country**		**Citizenship**	
Post Office Address							
Post Office Address							
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature						**Date**	
Residence: City		**State**		**Country**		**Citizenship**	
Post Office Address							
Post Office Address							
City		**State**		**ZIP**		**Country**	

SEQUENCE LISTING

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